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**Original Article**

# Colonizing Pneumococcal Serotypes in Pakistani Children (<3 years) before the Introduction of Pneumococcal Conjugate Vaccine - Implications for Vaccine Formulation

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## Abstract

**Background:** Pneumococcal vaccine prevents pneumococcal infections by inhibiting pneumococcal colonization in the host. As pneumococcal vaccine covers limited pneumococcal serotypes, hence knowledge of prevalent pneumococcal serotypes in a population is critical to estimate the effectiveness of vaccine in that population. This is the first study from Pakistan that reports colonization rates and colonizing pneumococcal serotypes in Pakistani children during pre-vaccination era.
**Study design, settings and duration:** A cross sectional study was conducted at PHRC Research Centre, Jinnah Postgraduate Medical Centre (JPMC), Karachi in collaboration with EPI Centre, National Institute of Child Health (NICH), Karachi during one year.
**Materials and Methods:** The study was conducted in continuation with a previous study in which nasopharyngeal swabs of healthy children coming to NICH for the EPI vaccination were collected and cultured for the presence of *S. pneumoniae*. Pneumococcal isolates were stored frozen in skimmed milk tryptone glucose glycerol (STGGA) medium. These isolates were then revived and serotyped using multiplex PCR as per the recommendations of Centre for Disease Control (CDC), USA to find out if the serotypes identified are being covered by recently introduced pneumococcal vaccine.
**Results:** Out of total, 61 (31%) pneumococcal isolates that were recovered from nasopharyngeal swabs of 192 healthy children, 58 (95.1%) isolates could be revived and were serotyped. The most prevalent serogroup in was identified to be 6A/6B/6C/6D which was isolated from 9 (15.5%) of children followed by 19F, 4 (6.9%). Both serotypes were covered by 10-valent pneumococcal vaccine. Overall 23(39.7%) isolates were covered by 10-valent vaccine and 26 (44.8%) by 13-valent pneumococcal conjugate vaccine. Predominant serotypes or serogroups in our population that were not being covered by both of these vaccines were 13F, 10F/10C/33C and 38/25F/25A, each was isolated from 4(6.9%) constituting 20.7% non-vaccine serotypes.
**Conclusion:** Serotype coverage of PCV-10 was low in Pakistani children which could be improved by using PCV-13.
**Policy message:** The findings will be be helpful 1) for authorities to identify the appropriate vaccine for our population and 2) for industry to formulate a vaccine with a serotype combination that may give desired results to prevent pneumococcal infections in our population.
**Key words:** *S. pneumoniae*, pneumococcal conjugate vaccine (PCV), serotypes, nasopharyngeal colonization.

## Introduction

Streptococcus pneumonia is a major community acquired pathogen involved in various invasive (meningitis and septicemia) and non-invasive (pneumonia, sinusitis and otitis media) infections.[1] Pneumococcal infections contribute to high morbidity and mortality throughout the world.[2] It is estimated that pneumococcal disease claims 1.6 million lives each year worldwide.[3] Moreover, a large number of patients who survive undergo severe neurological sequelae including unilateral and bilateral deafness.[4] In Pakistan, an estimated10 million cases of pneumonia are observed each year and S.pneumoniae is found to be associated with 10% of cases.[5,6]

The persistent high burden of invasive and noninvasive pneumococcal infections and rapid emergence of multidrug resistant pneumococcal strains have contributed to the increased interest of health authorities towards preventive strategies against pneumococcal infections. Substantial work has been done towards the development of a vaccine which could reduce the morbidity as well as

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**Authors Contribution**

SB conceptualized the project, performed literature search and statistical analysis. SB, TRS & FH did the data collection. Drafting, revision and wrinting of manuscript was done by SB, FH & WA.

mortality associated with pneumococcal infections in last two decades. Currently three types of pneumococcal conjugate vaccines (PCV) are available including PCV10, PCV13 and 23-valent pneumococcal polysaccharide vaccines. These vaccines are designed on the basis of epidemiological data of diseases causing serotypes of Pneumoccocci in different parts of the world particularly in the western countries. Varied responses to pneumococcal vaccination programs in terms of efficacy have been reported among different populations which are attributed to the differences in ethnicity, crowding, environmental features and socioeconomic features.[3]

Government of Pakistan with the support of GAVI Alliance decided to introduced PCV10 in routine Expanded program for immunization (EPI) in 2013,[7] but since no data regarding serotype distribution of pneumococcal strains causing infection in Pakistani population is available so it was difficult to estimate that to what extent we may control pneumococcal infections if such a vaccine is introduced in Pakistan. In the absence of baseline data (regarding actual burden of pneumococcal infections, disease causing serotypes of *S. pneumoniae*, colonization rates and immune status of children) before the introduction of pneumococcal vaccine, it may not be possible to assess the efficacy of vaccination program in achieving desired results.

The key mechanism involved in the development of Pneumococcal disease is proposed to be the Pneumococcal colonization and it is established that Pneumococcal disease may never occur in the absence of preceeding colonization.[8] It is also claimed that Pneumococcal vaccine causes significant reduction in the nasopharyngeal carriage of pneumococci resulting in decreased morbidity and a significant reduction in Pneumococcal colonization rates after vaccination indicating the effectiveness of vaccination program.[9,10]

Current study was conducted as a continuity of a previous study which was done to determine the colonization rates among healthy children and their immune status against *S. pneumoniae* prior to vaccine introduction.[11] The present study determined the serotypes of Pneumococcal strains isolated from these children to identify that whether these serotypes were covered by the Pneumococcal vaccine (PCV10) being used in Pakistan.

## Materials and Methods

The study was conducted at PHRC Research Centre, Jinnah Postgraduate Medical Centre (JPMC), Karachi in collaboration with EPI Centre, National institute of child health (NICH), Karachi. All the laboratory work was carried out in microbiology and molecular biology department of PHRC Research Centre, JPMC, Karachi.

Total 61 pneumococcal isolates that were isolated from the nasopharyngeal cultures of 192 healthy children via already conducted study were included in the study.[11] A sample size of 191 children (<3 years of age) had been calculated at 7% precision and 95% confidence interval on the basis of previous study which showed that 55% of infants were colonized with pneumococci.[12]

Healthy children attending EPI centre of NICH to receive routine immunization were enrolled in the study after taking informed consent from their parents.

Children with chronic or recurrent pulmonary disease, those who had received antibiotics in last seven days, history of hospitalization for more than 1 week or who had received pneumococcal vaccine were excluded from the study.

A predesigned structured proforma was used to record patient's demographics, history of any illness, crowding at home or exposure to passive smoking.

Nasopharyngeal swabs of children were collected using flexible twisted wire Dacron cotton tipped swabs (ProLab, Canada). These swabs were immediately put in sterile STTGA medium, vortexed and transported to the Microbiology lab of PHRC Research Centre, JPMC Karachi as per WHO guidelines. Culture of these swabs was done on blood agar supplemented with 5-7% sheep blood and plates were incubated at 37°C for 24-48 hours. Identification of Pneumococcus was done on the basis of colonial morphology and optochin susceptibility test.

Pneumococcal isolates were saved at -40°C in Skimmed milk tryptone glucose glycerol (STGGA)

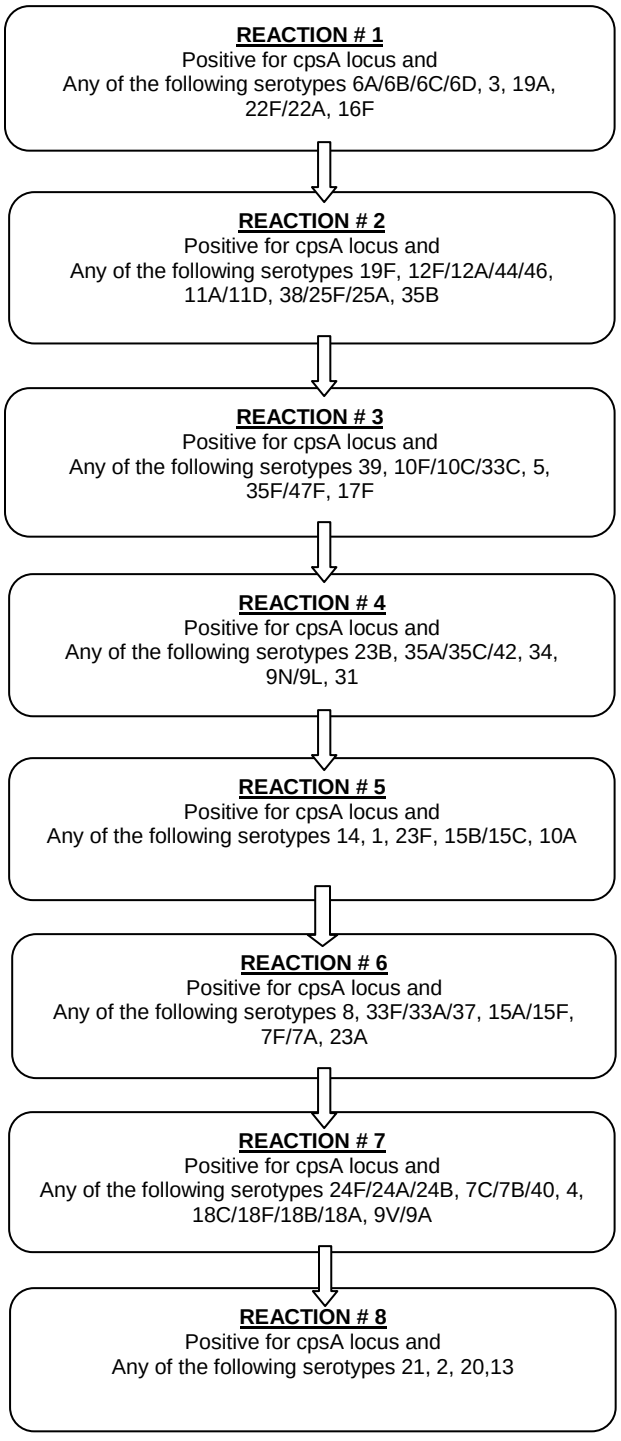


**Figure 1: Sequential multiplex PCR scheme used for pneumococcal serotyping.**

medium. Stored isolates were revived in BHI broth and subcultured on blood agar (oxoidTM). DNA extraction was carried out using DNA kit for bacterial samples (Bio Basic Inc. Canada) as per manufacturer's guidelines. DNA extraction for isolates that did not grow on subculturing was performed directly from STTGA vial, taking 200ul STTGA as sample. These extracts were only considered if the control band i.e. cpsA was visible on the gel after amplification.

Extracted DNAs were kept frozen at -40oC till further use for Multiplex PCR. Multiplex PCR amplification was performed as per the method described by Centre for Disease Control (CDC) USA (CDC. 2014). Briefly multiplex PCR was performed in 25ul reaction volume using 2 x multiplex PCR master mixes (Qiagen Inc. Valencia, CA), recommended concentrations of each of the 6 or 5 pairs of primers including a pair of primer for cpsA locus (0.5uM) as described previously,[13] and 2ul of DNA. PCR reaction was then performed using LabNet (USA) gradient system under following conditions; 95°C for 15mins followed by 35 cycles of amplification (94°C for 30 seconds, 54°C for 90 seconds and 72°C for 60 seconds) and then a final hold of 10 minutes at 72°C. Sequential multiplex PCR scheme that we followed is depicted in Figure-1. Amplified products were analyzed through gel electrophoresis using ethidium bromide stain under UV trans-illuminator using 100bps DNA ladder for measuring the size of DNA bands.

Thirty nine control strains (provided by CDC, USA) including all 23 serotypes incorporated in the 23 valent pneumococcal vaccine were run as controls to validate the primers and PCR protocol. Five controls for the respective five serotypes included in each multiplex PCR reaction were run with each batch except for the reaction eight which covers four serotypes.

The study was approved by the ethical review committee of National Institute of Child Health, Karachi.

The data feeding and analysis was done on computer package SPSS (Statistical Packages for Social Sciences) version 19.0 to determine the clinical characteristics in terms of frequencies and percentages for categorical variables (colonization rates, gender, Pneumococcal serotypes.) whereas mean ± SD was calculated for numerical variables (age).

## Results

A total of 192 children were enrolled in the study. The mean age of children was 15 ± 11 months. Ninety eight (51%) were male while 94 (49%) were female with ratio 1:1.

Nasopharyngeal swab culture for Pneumococcus was positive in 61 (31.7%) cases. Out of total 61 pneumococcal isolates, 58 (95.1%) isolates could be revived and were serotyped. The most prevalent sero-group in our population was

identified to be 6A/6B/6C/6D which was isolated from 9 (15.5%) children followed by 19F, isolated from 4 (6.9%).

Overall 23 (39.7%) isolates were covered by 10-valent vaccine and 26 (44.8%) by 13 valent pneumococcal conjugate vaccine. Predominant serotypes or serogroups in our population that were not being covered by both these vaccines were 13F, 10F/10C/33C and 38/25F/25A, each of these were isolated from 4 (6.9%) together constituting 20.7% non-vaccine serotypes.

Detailed results for serotyping along with details that whether these were included in 10 valent or 13 valent pneumococcal conjugate vaccines are given in Table.

**Table: Colonizing pneumococcal serotypes in children (<3 years).**

| *Serotypes* | *Isolation N (%)* |
|---|---|
| 6A/B/C* | 9(15.5) |
| 19F* | 4 (6.9) |
| 13F | 4 (6.9) |
| 38/25F/25A | 4 (6.9) |
| 10F/10C/33C | 4 (6.9) |
| 19A** | 3 (5.2) |
| 16F | 3 (5.2) |
| 14* | 2 (3.5) |
| 23F* | 2 (3.5) |
| 15B/15C | 2 (3.5) |
| 10A | 2 (3.5) |
| 9V* | 1 (1.7) |
| 3** | 1(1.7) |
| 23A | 1(1.7) |
| 11A/11D | 1 (1.7) |
| 21F | 1 (1.7) |
| 35A/35C/42 | 1 (1.7) |
| 3F | 1 (1.7) |
| 22F/22A | 1 (1.7) |
| 15A/15F | 1 (1.7) |
| 7* | 1 (1.7) |
| Non-typeable | 7 (12.1) |
| Total | 58 (100) |

*serotypes included in PCV-10 & PCV 13
** serotypes included in PCV-13 only

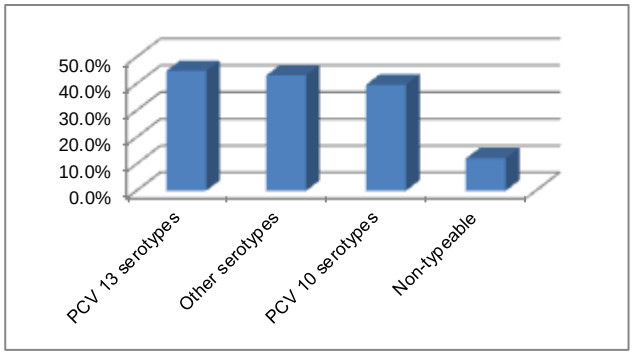


**Figure 2: Distribution of pneumococcal serotypes according to vaccine formulations.**

Figure-2 summarizes the distribution of serotypes as per vaccine formulations.

## Discussion

Present study is the first that reports the colonizing pneumococcal serotypes in young children during the pre-vaccination era in order to estimate the probable benefit of recently introduced pneumococcal vaccine in our population.

Nasopharyngeal carriage rate of 32% was observed in our study in healthy Pakistani children (<3 years), prior to the inclusion of pneumococcal vaccine in EPI program. Studies from neighboring countries show almost similar results. A nasal carriage rate of 47% was reported from Bangladesh among children <5 years of age.[14]

Present study indicates that only 40% colonizing serotypes in our population were covered by currently available PCV-10 suggesting a lower coverage rate as compared to United States (>74%) during the pre-vaccination era.[15] However, similar or even lower coverage rate of pneumococcal vaccine against carriage serotypes (i.e. 30%) was reported from Bangladesh.[14] Our results indicate a slightly lower coverage of PCV-10 as compared to a recent study reported from Karachi, Pakistan which showed 67% serotype coverage rate of PCV-10 in children <5 years of age.[16] However, Shakoor et al reported only those serotypes involved in children with complicated illness. Studies suggest that serotypes involved in carriage are more diverse than those involved in meningitis.[17] Similar coverage rates have been reported from Brazil where only 52% colonizing serotypes were included PCV-10 serotypes.[18]

Serogroup 6A/6B/6C/6D is identified to be the most prevalent followed by serotype 19F in our population in the pre-vaccination era. Both serotypes are covered by PCV-10. A study from United States revealed that the highest prevalence of these serotypes before the introduction of pneumococcal conjugate vaccine in 1999-2000.[19] A study from Sudan also showed the highest carriage rates of this sero-group in infants.[20] Same serogroup i.e. 6A/6B/6C/6D was identified as being predominantly responsible for invasive disease in children <15 years from South Saharan Africa [21] and Romania.[22]

Our study further identified a high proportion of serogroups/ serotypes 13F, 38/25F/25A, 10F/10C/33C, 19A and 16F in our children. Among these none of the serotype is covered by PCV-10. These serotypes together constituted total 31% pneumococcal carriage isolates in the present study. This means that incorporating these

serotypes in PCV-10 may increase the coverage rate of pneumococcal vaccine to 71%. However, an important consideration here may be that Shakoor et al from Karachi did not report isolation of 13F, 38/25F/25A and 16F in their study.[16] Therefore, it is important to provide evidence for involvement of these serotypes in disease causation before making any recommendation for vaccine.

Another finding that may be pertinent to mention here is that we did not isolate serotypes 4, 5 and 18C in our study which are included in PCV-10. Serotypes 4 and 18 have not been reported either from India [23] however, Bangladesh [14] did report serotype 5 only as rare serotype. This finding suggests that these serotypes may be replaced by some prevalent serotypes in the vaccine but contrary to our findings these serotypes though uncommon but have been reported by Shakoor et al in IPD cases.[16]

Considering the other available pneumococcal conjugate vaccine PCV-13 which incorporates 13 pneumococcal serotypes, present study showed a coverage rate of 45% carriage serotypes, which suggests approximately 5% increase in the coverage as compared to the PCV-10. Shakoor et al also reported an insignificant increase in coverage of IPD cases by PCV-13 as compared to PCV-10 in children below 5 years.[16]

This is the first study that has reported the pneumococcal serotype distribution among nasopharyngeal carriers of children in our population. The study suggests that the coverage rate of currently used pneumococcal conjugate vaccine (PCV-10) against carriage serotypes is not up to the mark. Different vaccine formulations employing the prevalent pneumococcal serotypes as suggested by the study may yield better coverage and results. However, the impact of currently used vaccine also needs to be studied to evaluate the actual benefit of the vaccine which is now in use for almost three years. The present study on carriage and Shakoor et al's study for IPD cases may be used as a reference to compare the findings of pre and post vaccination era.[16]

It was not a population based study rather - children were recruited from the EPI clinic hence it may not be representative of the whole Pakistani population. It was due to the fact that as we planned the study, when pneumococcal vaccine was introduced. However, since Karachi is a cosmopolitan city and the site selected for study invites all sort of people from different socioeconomic and ethnic groups hence the problem may be considered abridged.

Serotype coverage of PCV-10 vaccine is low in Pakistani children which may be increased by using PCV-13. Further studies on clinical isolates of pneumococci can guide to the right serotype combination for vaccine in our population.

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**Conflict of interest:** None declared.

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